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14. ABSTRACT
The point of our study is to analyze the immune mechanisms contributing to rejection of vascularized composite allografts (VCA) using murine models, and to try and overcome these immune responses and promote long-term VCA survival.

15. SUBJECT TERMS- nothing listed

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Table of Contents

1. INTRODUCTION

The point of our study is to analyze the immune mechanisms contributing to rejection of vascularized composite allografts (VCA) using murine models, and to try and overcome these immune responses and promote long-term VCA survival.

2. KEYWORDS

Vascularized composite allografts, allograft rejection, tolerance, costimulation blockade

3. OVERALL PROJECT SUMMARY

Our goals for the first year were: **CY13 Goals** Obtain regulatory approval and establish a murine hind-limb heterotopic model (Task 1) was completed. **CY14 Goals** Target key chemokine/receptor pathways promoting VCA rejection (Task 2); Begin initial phases of tasks 3 and 4; Task 3 is to test if peri-transplant costimulatory blockade will allow long-term VCA survival without development of chronic injury; Task 4 will test ability of Treg-based therapies to promote VCA outcomes.

Task 1 was completed, and led to works on Tasks 2 and 3 as will be summarized next.

TASK 2 - <u>Test effects of anti-CXCR3 targeting in VCA recipients</u>

This was undertaken given our qPCR data, noted in the previous quarterly report, of increased CXCR3 mRNA in VCA samples undergoing rejection in unmodified recipients, with a rise by day 3 and peaking at day 5 post-transplant (Tx). We treated recipients with a hamster anti-CXCR3 monoclonal antibody (mAb) (CXCR3-173) we had generated and previously shown was highly effective in prolonging cardiac and islet allograft survival, especially when combined with a sub-therapeutic regimen of rapamycin (RPM) (1).

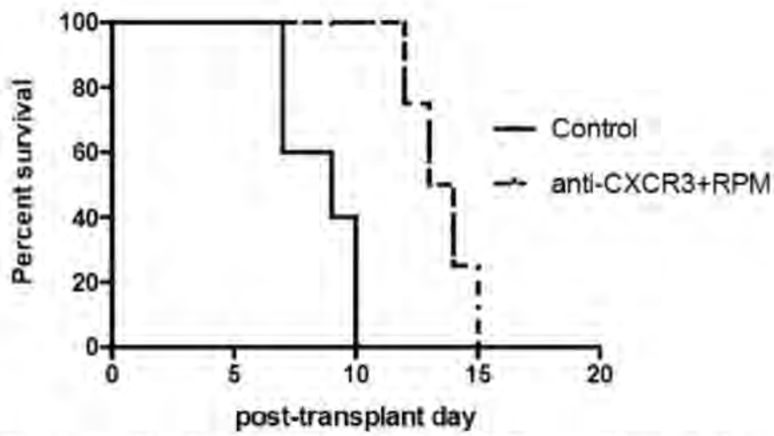

Fig. 1. Effects of anti-CXCR3 mAb plus RPM on VCA survival.

VCA recipients were treated with 4 doses of an anti-CXCR3 mAb 200 µg/day, qod, from the day of transplantation, plus 0.2 mg/kg/day of RPM, i.p., for a total of 14 days **(Fig. 1)**. This therapeutic regimen prolonged VCA survival ($p<0.05$), but was far less effective than anticipated. In our previous studies, optimal effects (i.e. permanent allograft survival) after brief peri-transplant therapy) were seen using 500 µg qod along with 0.1 mg/kg/day of RPM for 14 days from the time of transplantation (1).

TASK 3 - <u>Test effects of costimulation blockade using CD154 mAb/DST plus CTLA4Ig</u>

Previous studies from our group showed that use of MR-1 mAb directed against CD40L (CD154) was highly effective at prolonging cardiac allograft survival in mice, especially when coupled with donor splenocyte transfusion (DST) at the time of transplantation (2,3). We have also shown that therapy with CTLA4-Ig was effective at prolonging cardiac allograft survival. Lastly, and remarkably, the combination of these 2 approaches, i.e. CD154 mAb and CTLA4Ig, was able to promote long-term skin allograft survival (4). We have now tested these approaches with the heterotopic VCA hind-limb model in mice (**Fig. 2**).

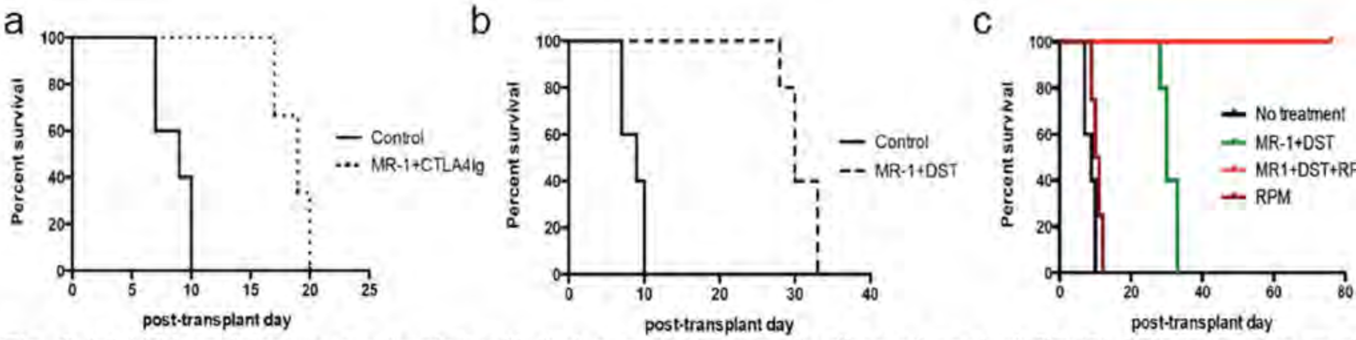

Fig. 2 *(a) VCA recipients were treated with 6 doses of MR-1 mAb, directed against CD40L (CD154), plus murine CTLA4Ig, using 500 µg of each, qod from transplantation, resulting in only a doubling of allograft survival ($p<0.05$). (b) VCA recipients were treated with MR-1 (CD154) mAb (200 µg) plus donor splenocyte transfusion (DST, 5 x 10^6 cells, i.v.) at the time of transplantation, leading to a tripling of allograft survival ($p<0.01$). (c) Addition of 2 weeks of rapamycin (RPM, 2 mg/kg/d, i.p.) to MR1/DST protocol has led to long-term VCA survival (>90 days at the time of submission of this report).*

Combined use of CD154 mAb (MR-1) and CTLA4Ig only increased allograft survival by about 2-fold (**Fig. 2a**). These data were surprising to us. We had anticipated that combined costimulatory blockade (CD154 mAb/CTLA4Ig) would be much more effective in achieving long-term VCA survival than was observed (**Fig. 2a**), given the published effects of this protocol on skin allograft survival in this same strain combination (BALB/c->C57BL/6) (4), and our assumption that the skin would be the most immunogenic component of the limb allograft. i.e. we hypothesized that if skin allograft rejection could be controlled, then the rejection of the other components of the allograft (subcutaneous tissues, muscle and bone) would likely also be suppressed. In separate studies, we will now test if this protocol is indeed effective in preventing skin allograft rejection in our hands, too,

In contrast to combined CD154 mAb (MR-1) and CTLA4Ig, use of a single peri-transplant injection of CD154 mAb plus DST was more effective than multiple injections of CD154 and CTLA4Ig, resulting in a tripling of allograft survival (**Fig. 2b**). The latter therapy results in intravenous trafficking of donor cells to the spleen where they are largely trapped and provide a pulse of donor MHC to the recipient immune system. The concomitant administration of CD154 mAb is thought to prevent generation of a second signal and render the host alloreactive T cells anergic (2). These considerations led us to consider the testing, in future studies, of the effects of multiple rounds of CD154/DST, e.g. every 3 weeks, and also to propose that donor MHC+ cells within donor bone marrow might provide an ongoing stimulus for rejection (see next section)?

Fig. 3 *Well functioning VCA at 90 d post-Tx (single MR-1/DST plus 14 d of RPM).*

Lastly, the best effects to date were seen by adding 14 days of RPM (2 mg/kg/d, i.p.) to the CD154 mAb/DST protocol, with engraftment, as of submission, of >90 days (**Fig. 2c**) and healthy appearance (**Fig. 3**). At >100 days, these animals will be tested for evidence of tolerance induction (acceptance of a second donor transplant but rejection of third party graft).

Can donor bone-associated tissue(s) contribute to VCA survival?

We have made 2 observations concerning the importance of donor bone-associated tissues on VCA survival in recipients receiving costimulation blockade therapy (**Fig. 4**). First, gamma-irradiation of donor allografts pre-

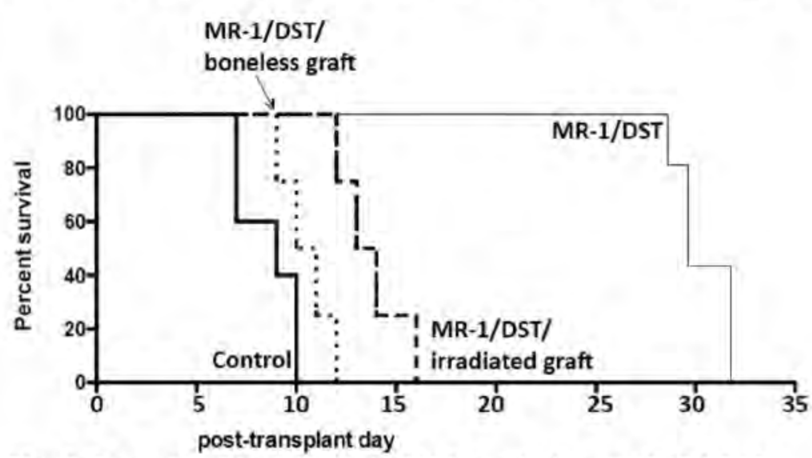

Fig. 4*. Donor bone contributes to VCA survival post-CD154/DST.*

transplant significantly shortened survival of heterotopic allografts in mice treated with a single injection of CD154 mAb (MR-1)/DST ($p<0.05$). Second, complete removal of the limb bone from the graft prior to transplantation shortened VCA survival even further in recipients treated with a single injection of CD154 mAb (MR-1)/DST.

While preliminary, these data suggest that a component of the bone is required for the efficacy of CD154/DST-based costimulation blockade in this model. One possibility is that donor bone marrow cells contribute to the maximal stimulation and T cell allo-activation that is optimally targeted by CD154 mAb. Irradiation likely kills the majority of donor marrow cells and the damaged or dead may not be efficiently targeted to lymphoid tissues, especially the spleen, though some may survive for a period and contribute, along with DST cells to host sensitization. However, complete removal of bone takes away all such cells, and reduces the synchronous delivery of signal one to recipient T cells. Alternately, a component of donor marrow may actually contribute to immunoregulation, e.g. donor Treg cells, B cells or myelomonocytic cells. These options remain to be explored, but as one example of development of this work, we have undertaken engraftment of hind limbs from immunodeficient Rag1-/- BALB/c donors into WT C57BL/6 recipients to test whether T or B cells play a role in the prolonged survival induced by CD154 mAb plus DST.

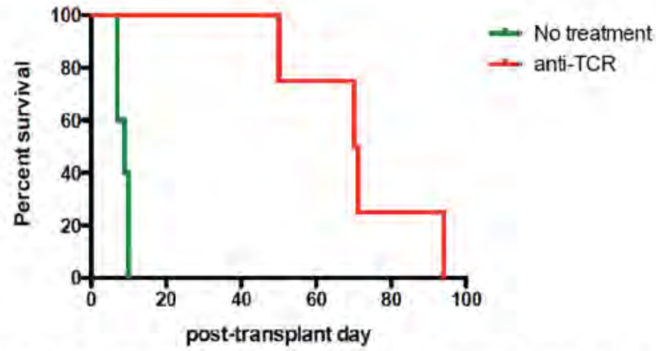

Fig. 5. Beneficial effect (p<0.01) of hamster anti-mouse TCRβ mAb (H57-597) on VCA survival. VCA recipients were treated with mAb using 100 µg qod until day 14 post-Tx (i.e. 8 doses).

Does peri-transplant lymphocyte deletion promote long-term VCA survival?

Induction therapy with polyclonal or monoclonal Abs is widely used in clinical transplantation. Two anti-thymocyte globulin (ATG) preparations are licensed for clinical use in the US for treatment of acute renal allograft rejection, and are used as induction agents before and/or during kidney transplantation. ATG drastically circulating reduces the number of circulating T cells, preventing acute cellular rejection of transplanted organs. Other transplant groups use CD25 (anti-IL-2R) mAb for induction therapy, given its safety profile and specificity for activated T cells.

Given this clinical rationale, we have tested the efficacy of a depleting anti-TCR mAb that we have previously shown was efficacious in murine cardiac allograft recipients (5). Anti-TCR mAb, given for 2 weeks from the time of engraftment and then stopped, prolonged VCA survival for ~70 days (50% survival data, **Fig. 5**), with excellent results; e.g. see healthy appearance and hair growth on 3 examples at 36 days post-Tx (**Fig. 6**).

Anti-TCR mAb depleted circulating T cells (CD4 and CD8) as shown by flow cytometric analysis of blood samples collected at day 14 post-Tx, whereas the proportions of CD4+Foxp3+ Treg cells were comparable (**Fig. 7**). Comparable depletion was still apparent at 36 days post-Tx (data not shown).

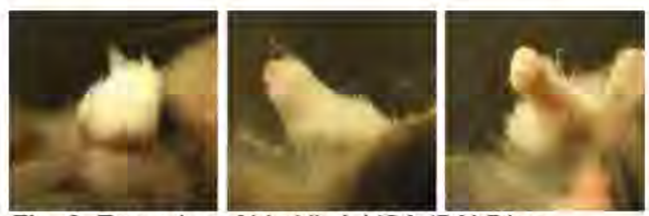

Fig. 6. Examples of hind limb VCA (BALB/c-> C57BL6) at day 36 post-Tx in recipients treated with a 2 weeks course of anti-TCR mAb, beginning at the time of engraftment.

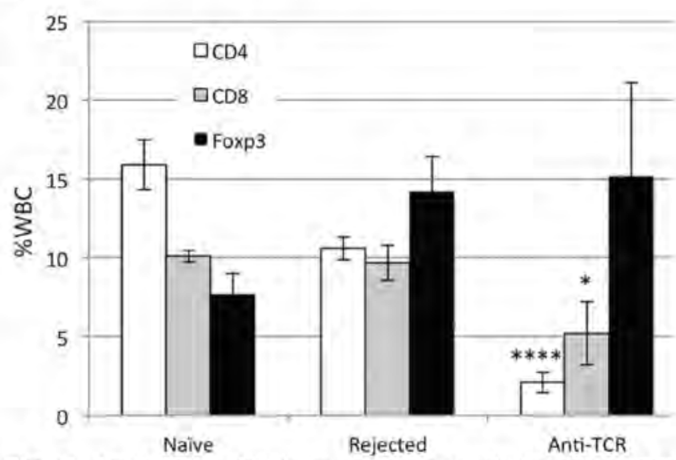

*Fig. 7. Anti-TCR mAb therapy decreased circulating CD4 and CD8 cells compared to untreated VCA recipients undergoing rejection, or normal controls; graph shows analysis of the % of circulating WBC at day 14 post-Tx for CD4+ and CD8+ cells (*p<0.05 and ****p<0.001 vs. rejection group). Data for Foxp3 refer to the proportion of Foxp3+ cells within the CD4 population in each case.*

Effects of regular immunosuppression

To test standard immunosuppressive therapy could achieve long-term VCA survival, we treated VCA recipients with FK506 (Tacrolimus) (**Fig. 8**). While 2 weeks of FK506 at a dose of 2 mg/kg/d resulted in a VCA survival of a little more than 3 weeks, therapy for 4 weeks at the same dose led to prolonged allograft survival, with 50% surviving for >100 days. ELISPOT analysis of recipient alloreactivity at 100 days post-Tx, with the readout of IFN-γ production in response to exposure of donor lymphocytes to recipient splenocytes *in vitro*, showed marked reductions in host alloresponses in FK506-treated recipients (**Fig. 9**). These data show that immunosuppression can largely erase sensitization and promote long-term survival, and will be used as a point of comparison with other successful therapies requiring only peri-transplant administration (e.g. anti-TCR mAb).

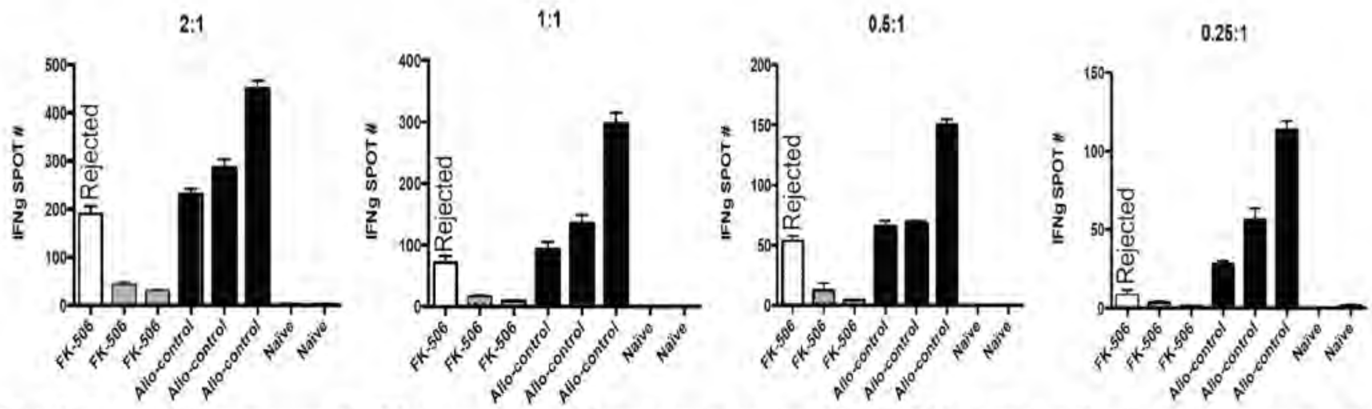

Fig. 9. ELISPOT responses at 100 d post in 3 VCA recipients treated with FK506 (2 mg/kg, 28 d), including one that rejected (white boxes) and 2 that were accepted long-term (grey boxes), plus 3 VCA recipients experiencing acute rejection (black boxes), and 2 naïve normal control mice.

4. KEY RESEARCH ACCOMPLISHMENTS

• Despite showing statistical significance, anti-CXCR3 mAb targeting was relatively ineffective in prolonging VCA survival.

• Anti-CD40L/DST/RPM therapy led to >100 d survival without maintenance immunosuppression.

• Anti-TCR mAb plus RPM (14 d) was also highly efficacious, achieving 70-80 d of VCA survival.

5. CONCLUSION

Long-term VCA survival is possible with brief peri-transplant therapy and without maintenance immunosuppression. We need to (i) test whether these animals are tolerant of second donor grafts but capable of rejecting third party grafts using this approach; (ii) assess the role of Tregs in this long-term outcome; and (iii) test whether this approach will also support orthotopic hind-limb VCA survival.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

None.

7. **INVENTIONS, PATENTS AND LICENSES**

None.

8. REPORTABLE OUTCOMES

None

9. OTHER ACHIEVEMENTS

None.

10. REFERENCES

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11. APPENDICES

Revised Quad Chart.

Positioning Vascularized Composite Allotransplantation in the Spectrum of Transplantation

CRMRP-JPC8, “Novel Immunomodulatory Therapies for Vascularized Composite Allotransplantation” MR120023P3

PI: Wayne W. Hancock Org: Children’s Hospital of Philadelphia & University of Pennsylvania Award Amount: $1,996,875

Study Aims

- Establish murine hindlimb transplant models
- Target chemokine/chemokine receptor pathways promoting VCA rejection
- Test if costimulation blockade will promote long-term VCA survival
- Test if Foxp3+ Treg-directed therapies will promote long-term VCA survival
- Test optimal combinations of therapies so as achieve VCA engraftment and function, as well as preventing development of chronic injury

Approach

Our combined group recognizes that the long-term effects of chronic immunosuppressive therapies, including increased rates of nephrotoxicity, atherosclerotic disease, diabetes and tumor formation, outweigh their usefulness in VCA recipients. To identify less toxic and more suitable therapies for management of VCA, the group will undertake basic science studies in murine models to elucidate the mechanisms of immune rejection of VCA, and test the efficacy of novel strategies to achieve long-term engraftment without use of maintenance immunosuppressive therapy.

Example of a well accepted hind-limb heterotopic VCA at 90 d post-Tx (stringent BALB/c->C57BL/6 model). Long-term allograft survival was achieved using a single injection of CD154 mAb and donor splenocyte transfusion (DST), plus 14 d of rapamycin therapy.

- **Ultimately**, we wish to achieve long-term survival and function of orthotopic hind limb allografts in mice (BALB/c->C57BL/6).
- **Accomplishment**: in our first year, we have achieved this for heterotopic limb allografts (**photograph**), using peri-operative therapies that are less toxic than the current types of maintenance immunosuppression used in organ transplant recipients.

Timeline and Total Costs (includes direct & indirect costs)

Activities	2013	2014	2015	2016
Task 1. Obtain regulatory approval and establish murine hindlimb models at CHOP				
Task 2. Target key chemokine/chemokine receptor pathways promoting VCA rejection.				
Task 3. Test if peri-transplant costimulatory blockade will allow long-term VCA survival without development of chronic injury.				
Task 4. Test ability of T-regulatory (Treg) based therapies to promote VCA outcomes.				
Task 5. Test optimal combinations of therapies based on data generated above.				
Estimated Budget (total $K)	511,875	495,000	495,000	495,000

Updated: October 14, 2014

Goals/Milestones

√**CY13 Goal** – We have established a VCA model and begun chemokine targeting (Task 1);

√**CY14 Goals** – We continuing chemokine/receptor targeting (Task 2), and using costimulation blockade, we have achieved considerable success (long-term engraftment with brief peri-Tx therapy);

CY15 Goal - Complete costimulation blockade & Treg studies (Tasks 3 & 4)

CY16 Goal - Undertake final studies using optimal combinations (Task 5)

Comments/Challenges/Issues/Concerns

- If timelines change, comment here: no comments
- If off by more than 1 quarter, comment here; no comments

Budget Expenditure to Date

Projected Expenditure: As budgeted

Actual Expenditure: As budgeted